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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C07K 14/47, A61K 38/17	A1	(11) International Publication Number: **WO 99/58566** (43) International Publication Date: 18 November 1999 (18.11.99)

(21) International Application Number: PCT/SE99/00807

(22) International Filing Date: 12 May 1999 (12.05.99)

(30) Priority Data:
9801695-9   14 May 1998 (14.05.98)   SE

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(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

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(54) Title: INHIBITION OF p53 FUNCTION BY A p53-DERIVED SUBSTANCE

(57) Abstract

The present invention provides a substance which has the property of inhibiting the sequence specific DNA binding activity of p53, said substance being based on the fragment of the DNA binding core domain of p53. The substance is shown to inhibit p53 DNA binding *in vitro* and transactivation function in living cells. The invention includes the use of these substances, and in screening for mimetics.

INHIBITION OF p53 FUNCTION BY A p53-DERIVED SUBSTANCE

Field of the invention

The present invention relates to a peptide derived from the apoptosis regu-
5   lating protein p53 as well as to different uses thereof.

Background

Apoptosis is a morphologically defined form of programmed cell death,
which has very recently been seen after ischemic injury to the central ner-
10   vous system and heart muscle and seems likely to be involved in .chronic
degenerative diseases like multiple sclerosis (Crowe et al., 1997). A protein
denoted p53 has been shown to be a key regulator of apoptosis in tissues of
different types. Under normal conditions, p53 is inactive and present at a
very low levels in a latent form. In cells, the p53 protein is activated and
15   detectable only under stress conditions, such as hypoxia and DNA damage
(Ko, L.J., and C. Prives. (1996) p53: "Puzzle and paradigm". Genes & Dev.
10, 1054-1072). Thus, accumulation of p53 in cells results in the induction
of apoptosis or growth arrest, depending on the cell type. Therefore, the ac-
tivity of the p53 protein is very tightly regulated, both through allosteric
20   mechanisms and protein levels.

It has been shown that p53 expression is increased in damaged neurons in
models of ischemia and epilepsy. Using p53 null mice, it was shown that
p53 expression is required for induction of cell damage in a model of seizu-
25   re activity (Morrison et al., (1996): "Loss of the p53 tumor suppressor gene
protects neurons from kainate-induced cell death", J. Neurosci. 16, 1337-
1345). In addition, loss of p53 function prevents cell death in the central
nervous system during embryonic development (Macleod et al., (1996):

"Loss of Rb activates both p53-dependent and independent cell death pathways in the developing mouse nervous system. EMBO J. 15, 6178-6188). Recent studies have demonstrated the direct involvement of p53 in apoptosis of neurons and oligodendrocytes (Eizenberg, O., A. Faber-Elman, E. Gottlieb, M. Oren, V. Rotter, and M. Schwartz. (1995): "Direct involvement of p53 in programmed cell death of oligodendrocytes". EMBO J, 14, 1136-1144; Eizenberg, O., A. Faber-Elman, E. Gottlieb, M. Oren, V. Rotter, and M. Schwartz. (1996): "p53 plays a regulatory role in differentiation and apoptosis of central nervous system"; Mol. Cel. Biol., 16, 5178-5185).

Taken together, these findings suggests that pharmacological regulation of p53 function would decrease the extent of tissue injury. Consequently, there is a need within this field to achieve substances and methods for use therein, which enable such a regulation.

For the above defined purpose, it has been shown that p53 is a specific DNA binding protein, which acts as a transcriptional activator of genes that control cell growth and death. Thus, the ability of the p53 protein to induce apoptosis is dependent on the specific DNA binding function. Mutant p53 proteins carrying amino acid substitutions in the core domain of p53, which abolish the specific DNA binding are unable to induce apoptosis in cells. Therefore, in order to obtain such substances and methods as defined above, an inhibition of p53 DNA binding is essential in order to inhibit p53-triggered apotosis in tissues during pathological conditions.

WO 95/19367 shows screening assays for identifying agents that affect p53-dependent expression. It is suggested that such agents are capable of interaction with p53.

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WO 93/24525 discloses a peptide comprising an amino acid sequence derived from human p53 protein useful in prophylactic or therapeutic treatment of diseases showing p53 overexpression.

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WO 97/14794 discloses that p53 is dependent on a C-terminal negative regulatory domain and how phosphorylation or deletion of said domain activates the DNA sequence-specific binding thereof. The aim of this patent is to obtain activation of latent p53 protein, and this is achieved by providing a

10 substance, which consists of the C-terminal regulatory domain of p53 protein or a fragment of a murine p53 including the epitope bound by antibody Pab241, which according to the WO 94/12202 is used to activate mutant p53.

15 The object of WO 95/17213 is to identify the major structural domains of the human p53 protein. The possibility that the DNA-binding domain of p53 resides in the C-terminal region thereof is discussed. It is suggested that the central, core domain contains the sequence-specific DNA binding activity of p53. Thus, this patent relates to a recombinant nucleic acid molecule

20 encoding a portion of the p53 protein. Said polypeptide binds specifically to DNA having the sequence specifically recognized by p53. It is also capable of activating transcription of the DNA sequences, whose transcription is activated by p53. The suggested use of this polypeptide is in a method for treating a subject suffering from a neoplasm associated with the presence of

25 mutant p53 protein.

Further, Anne M. Fourie et al (The Journal of Biological Chemistry: "HSP70 Binding Sites in the Tumor Suppressor Protein p53", Vol. 272, No.

31, August1, 1997) show that most of the binding sites for proteins be-
longing to the HSP70 family are present in the hydrophobic core of the
central DNA binding domain of a mutant p53. Thus, this reference relates to
finding of protein binding domains of mutant p53, i.e. an oncogenic form of
5   the protein. It is suggested that the C-terminal domain of p53 interacts with
the core domain thereof, and that a disruption of this interaction increases
the DNA binding activity of the core.

In summary, the prior art does not disclose any specific inhibitor of the
10   apoptosis inducing activity of p53 protein.

Summary of the invention
The object of the present invention is to fulfill the above defined need. This
is achieved by providing a substance capable of inhibiting the sequence
15   specific DNA-binding activity of latent p53 protein, as disclosed in claim 1.
The substance according to the invention is used in pharmaceutical compo-
sitions intended for treatment of pateints suffering from various degenerati-
ve conditions.

20   Brief description of the drawings
Figure 1A-C illustrates how the peptide according to the invention inhibits
the sequence specific DNA binding of the p53 protein.
Figure 2 discloses how the DNA binding of the constitutively activated
GST-p53-Δ30 fusion protein is inhibited by the peptide according to the in-
25   vention.
Figure 3A-D illustrates the interaction of the peptide according to the in-
vention with the core and C-terminal domains of p53.

Figure 4A-B describes how the peptide according to the invention prevents the complexing of peptide 46 with the p53 core domain.

Figure 5A-B are diagrams illustrating the inhibition of the transactivation function of p53 by the peptide according to the invention.

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Definitions

In the present invention, the following terms are used as disclosed below:

"Peptide 14" is sometimes used to denote the peptide defined by SEQ ID
10   NO 1 of the present application. In analogy therewith, the peptides defined by SEQ ID NO 2, SEQ ID NO 3 and SEQ ID NO 4 are sometimes denoted peptide 13, peptide 15 and peptide 21, respectively.

A "derivative " is a peptide modified by varying the amino acid sequence of
15   the original p53 fragment. Such derivatives of the natural amino acid sequence may involve insertion, deletion or substitution of one or more amino acids, without fundamentally altering the essential activity of the peptide.

20   A "functional mimetic" means a substance which may not contain a fragment or active portion of p53 amino acid sequence, and which preferably is not a peptide, but which has some or all of the properties of the p53 fragment, in particular the property of inhibiting the specific DNA binding activity of p53 by binding thereto. More specifically, a preferred functional
25   mimetic may be a peptidomimetic, wherein a part or all of the peptide is replaced by a structure lacking peptide bonds. Whether completely or partially non-peptide, such a functional mimetic according to the invention will provide a spatial arrangement of reactive chemical moieties that closely re-

sembles the three dimensional arrangement of active groups in the peptide according to the present invention. As a result of this similar active-site geometry, the functional mimetic has effects on biological systems which are similar to the biological activity of the original molecule.

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A "functional moiety" means a non-p53 derived molecule, for example a label, a drug, or a carrier molecule.

The term "label" as used herein means a moiety, which has been joined,
10 either covalently or non-covalently, to the present substance in order to provide a detectable signal. Thus, such a "label" may be detected by spectroscopic, photochemical, biochemical, immunochemical, or chemical means. For example, useful labels include ^{32}P, fluorescent dyes, electron-dense reagents, enzymes (e.g., as commonly used in a ELISA), biotin, dioxigenin, or
15 haptens and proteins for which antisera or monoclonal antibodies are available (e.g., the peptide of SEQ ID NO 1 can be made detectable, e.g., by incorporating a radio-label into the peptide, and used to detect antibodies specifically reactive with the peptide).

20 The term "hybridising specifically to", refers to the binding, duplexing, or hybridising of a molecule only to a particular nucleotide sequence under stringent conditions when that sequence is present in a complex mixture (e.g., total cellular) DNA or RNA. The term "stringent conditions" refers to conditions under which a probe will hybridise to its target subsequence, but to no other sequences. Stringent conditions are sequence-
25 dependent and will be different in different circumstances. Longer sequences hybridise specifically at higher temperatures. Generally, stringent conditions are selected to be about 5°C lower than the thermal melting point Tm for the specific sequence at a defined ionic strength and pH. The Tm is the temperature (under defined ionic strength,

pH, and nucleic acid concentration) at which 50% of the probes complementary to the target sequence hybridize to the target sequence at equilibrium. (As the target sequences are generally present in excess, at Tm, 50% of the probes are occupies at equilibrium). Typically, stringent conditions will be those in which the salt concentration is
5   less than about 1.0 M Na ion, typically about 0.01 to 1.0 M Na ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30°C for short probes (e.g., 10 to 50 nucleotides) and at least about 60°C for long probes (e.g., greater than 50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide.

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The term "nucleic acid" refers to a deoxyribonucleotide or ribonucleotide polymer in either single- or double-stranded form, and unless otherwise limited, encompasses known analogs of natural nucleotides that can function in a similar manner as naturally occurring nucleotides.

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The term "antibody" refers to a polypeptide substantially encoded by an immunoglobulin gene or immunoglobulin genes, or fragments thereof which specifically bind and recognize an analyte (antigen).

20   Detailed description of the invention
Accordingly, in a first aspect, the present invention relates to a substance capable of inhibiting the sequence specific DNA-binding activity of p53 protein. The substance comprises a fragment of about 15-30, preferably about 20-25 and most preferred about 22, amino acids of the core domain
25   of p53, or a functional mimetic of said fragment. Thus, it is to be understood that said fragment need not be identical to the sequence of the core domain of p53, but may include variations, as long as the activity thereof is preserved. Thus, said fragment may also be a derivative of the p53 sequen-

ce, or an active portion thereof. In this context, an "active portion" means a portion of the p53 peptide which is less than the full amino acid sequence of the fragment above, but which retains the property of inhibiting the specific DNA binding activity of p53. Also it is to be understood, that in the present

5   application, the human p53 is particularly preferred, even though p53 molecules of other origins may also be contemplated.

Thus, even though WO 93/24525 discussed above suggested that amino acid sequences derived from human p53 protein may be useful in the treat-

10   ment of disorders including an overexpression of p53, the present invention is the first to specify that the above defined substances are capable of exerting such an effect by the inhibition of the sequence specific DNA-binding activity of the p53 protein.

15   More specifically, the substance according to the invention is capable of providing said inhibition of the sequence specific DNA-binding activity of latent p53 by binding to the core domains and/or C-terminal domains of p53 protein. Thus, even though WO 95/19367 discussed above suggested binding to the DNA-binding region of p53 may influence the expression of

20   p53, the specific domains have never been identified prior to the present invention.

In a preferred embodiment of the invention, the fragment or functional mimetic thereof is coupled to a functional moiety, which enhances the p53 in-

25   hibiting effect of said substance. As mentioned above, such a moiety may be any non-p53 derived molecule, for example a label, a drug, or a carrier molecule. In one embodiment, the functional moiety is a carrier molecule

coupled to the present substance. In an alternative embodiment, the functional moiety is a p53 inhibiting molecule.

Thus, in one embodiment, the present substance is coupled to a label, pro-
5   viding a detectable signal. A wide variety of labels and conjugation techniques are known and reported extensively in both the scientific and patent literature. Suitable labels include radionucleotides, enzymes, substrates, cofactors, inhibitors, fluorescent moieties, chemiluminescent moieties, magnetic particles and the like.

10

In the most preferred embodiment of the invention, the present substance contains a fragment comprising a substantial part of the sequence disclosed in SEQ ID NO 1, preferably all of it. SEQ ID NO 1 discloses a 22-mer peptide, which is derived from the p53 core domain and corresponds to resi-
15   dues 105-126. This peptide is sometimes also denoted peptide 14.

As regards the preparation of the present substance, general methods are found e.g. in Sambrook et al. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Vols. 1-3, Cold Spring Harbor Laboratory. Once expressed,
20   the molecules according to the invention can be purified according to standard procedures in the art, including HPLC purification, fraction column chromatography, gel electrophoresis and the like, see generally Scopes, *Protein Purification*, Springer-Verlag, NY, 1982. Alternatively, standard chemical peptide synthesis methods may be used, see e.g. Barany and
25   Merrifield, *Solid-Phase Peptide Synthesis*: pp. 3-284 in *The Peptides: Analysis, Synthesis, Biology*. Vol. 2: *Special Methods in Peptide Synthesis*, Part A, Merrifield et al. J. Am. Chem. Soc., 85:2149-2156 (1963) and Stewart et al., *Solid Phase Peptide Synthesis*, 2nd ed. Pierce Chem. Co., Rockford, Ill.

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(1984). For the preparation of functional mimetics, techniques for the development of novel compositions of higher potency and/or greater bioavailability and/or greater stability than the original peptide can be found e.g. in Dean (1994), *BioEssays*, 683-687; Cohen and Shatzmiller (1993), *J. Mol.*

5 *Graph.*, 11: 166-173; Wiley and Rich (1993), *Med. Res. Rev.*, 13:327-384; Moore (1994), *Trends Pharmacol. Sci.*, 15:124-129; Hruby (1993), *Biopolymers*, 33:1073-1082; Bugg et al. (1993), *Sci. Am.*, 269:92-98.

The above mentioned WO 95/17213 relates to molecules binding to the sa-

10 me DNA as p53 does, whereby the transcription thereof may be activated. Thus, even though it relates to p53-based polypeptide fragments, WO 95/17213 solves another problem than the present invention by use of different molecules.

15 WO 97/14794 also relates to the problem of how to activate the sequence specific DNA binding activity of latent p53. To obtain this, a fragment of the C-terminal regulatory domain of p53 is used. Thus, the function of the p53 fragments disclosed in said patent application is the opposite from the object of the present invention, which is illustrated by the use of a fragment

20 from another domain than the p53 fragment forming the basis of the present invention.

Accordingly, a short synthetic peptide, peptide 14, derived from the p53 core domain, has been identified, which can be used to inhibit the specific

25 DNA binding function of p53. The inhibition of p53 function can be achieved in living cells upon introduction of the peptide in cells by lipopfection, i.e. transfection performed with a liposomal transfer compound, such as DOTAP (Boehringer-Mannheim), LIPOFECTIN etc. In addition, it has also

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been found that the peptide 14 binds to the core and C-terminal domains of p53. Peptide 14 is shown to inhibit p53 DNA binding *in vitro* and the transactivation function of p53 in living cells.

5   In a second aspect, the present invention relates to a pharmaceutical composition comprising a substance according to the invention, and as defined above, together with a pharmaceutically acceptable carrier. The pharmaceutical compositions according to the invention, and for use in accordance with the invention, may comprise, in addition to one of the above substan-

10   ces, a pharmaceutically acceptable excipient, buffer or stabilizer, or any other material well known to those skilled in the art and appropriate for the intended application. Such materials should be nontoxic and should not interfere with the efficacy of the active ingredient. Examples of techniques and protocols to this end may e.g. be found in Remington's Pharmaceutical

15   Sciences, 16th edition, Osol, A. (ed.), 1980.

The composition according to the invention may be prepared for any route of administration, e.g. oral, intravenous, cutaneous or subcutaneous, nasal, intramuscular or intraperitoneal. The precise nature of the carrier or other

20   material will depend on the route of administration. For a parenteral administration, a parenterally acceptable aqueous solutions is employed, which is pyrogen free and has requisite pH, isotonicity and stability. Those skilled in the art are well able to prepare suitable solutions and numerous methods are described in the literature (for a brief review of methods of drug deli-

25   very, see Langer, *Science* 249:1 527-1533 (1990)). Preservatives, stabilizers, buffers, antioxidants and/or other additives may be included, as required. Dosage levels can be determined by those skilled in the art, taking into account the disorder to be treated, the condition of the individual patient,

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the site of delivery, the method of administration and other factors. Examples of the techniques and protocols mentioned above can be found in Remington's Pharmaceutical Sciences, 16th edition, Osol, A. (ed), 1980.

5   In another embodiment, the composition according to invention further comprises one or more additional p53 inhibitors.

A third object of the present invention is the use of substance defined above as a medicament. In particular, the invention relates to the use of these sub-

10   stances as medicaments for minimizing the hypoxic tissue injury in myocardial infarction and stroke and for the treatment of degenerative and autoimmune disorders caused by deregulated excessive apoptosis, such as multiple sclerosis, ischemia, epilepsy, neurodegenerative diseases, anaplasitic anemia and others. Consequently, the present invention also relates to

15   the use of the above defined substances in the manufacture of a medicament for the treatment and/or prevention of degenerative diseases or conditions linked to enhanced deregulated apoptosis, such as the ones defined above, preferably ischemia or multiple sclerosis.

20   A fourth aspect of the present invention is a nucleic acid encoding such a fragment as the one present in the substance according to the invention. As the skilled man easily realises, the present invention encompasses all the possible variations that when expressed yield the present fragment. The nucleic acid may e.g. be DNA or RNA. The present invention also relates to

25   a vector comprising a nucleic acid according to the invention. Said vector may be a plasmid, a virus, *etc*, and is easily prepared by conventional methods disclosed in the literature. Thus, it is possible to produce the peptides according to the invention recombinantly by introducing a vector according

to the invention in procaryotic or eucaryotic cells, for example E. coli. Consequently, the invention also encompasses a cell comprising a nucleic acid encoding a peptide according to the invention.

5   A further aspect of the present invention is an antibody raised against the peptide 14 region of p53 or raised against peptide 14. Such an antibody may be monoclonal or polyclonal. Methods for producing antibodies against specific sequences are also disclosed in the literature (see e.g. Stites et al. (eds.), *Basic and Clinical Immunology* (4th ed.) Lange Medical Publications,

10   Los Altos, CA). The antibody may be produced in a cell, which cell then is also within the scope of the present invention. Further, selection may be performed in libraries of recombinant antibodies in phage or similar vectors (see e.g. Huse et al., *Science* 246:1275-1281; and Vaughan et al. (1996), Nature Biotechnology, 14:309-314).

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Another aspect of the invention is a method of screening for compounds capable of inhibiting the sequence specific DNA-binding activity of latent p53 protein, wherein a substance according to the invention is used. The present substance may be used in methods screening for: compounds having one or

20   more of the biological activities of the substance described above or compounds which are binding to the same site on a p53 molecule. Conventionally, the candidate compounds can be selected from a synthetic combinatorial library. Examples of screeing procedures for mimetics or binding partners include:

25   A. immobilizing the substances on a solid support and exposing the support to a library of labelled peptides or other candidate compounds, and detecting the binding of the peptides or candidate compound to the substances;

B. using the substances and a library of unlabelled candidate compound or peptides to find candidate compounds that compete or synergise with the substances in the inhibition of p53;

C. other combinations of solid phase substrates and binding
5   measurments;

D. using the substances and/or candidiate compounds in cell systems to determine whether the fragments or candidate compounds inhibit p53 activity.

10   A further aspect of the invention is method of designing an organic compound capable of inhibiting the sequence specific DNA-binding activity of p53 protein, wherein a substance according to the invention is used as a "lead" compound. In a preferred embodiment thereof, the organic compound is modelled to resemble the three dimensional structure of amino acids
15   no. 105-126 of p53 protein, i.e. the sequence of SEQ ID NO 1, or a shorter fragment of this region, which preferably exhibits substantially the same activity.

The designing of mimetics to a known pharmaceutically active compound is
20   a known approach to the development of pharmaceuticals based on a "lead" compound. This might be desirable where the active compound is difficult or expensive to synthesise or where it is unsuitable for a particular method of administration, e.g. peptides are unsuitable active agents for oral compositions as they tend to be quickly degraded by proteases in the alimentary
25   canal. Mimetic design, synthesis and testing are generally used to avoid randomly screening large number of molecules for a target property.

There are several steps commonly taken in the design of a mimetic from a compound having a given target property. Firstly, the particular parts of the compound that are critical and/or important in determining the target property is determined. In the case of a peptide, this can be done by systematically varying the amino acid residues in the peptide, e. g. by substituting each residue in turn. These parts or residues constituting the active region of the compound are known as its "pharmacophore".

Once the pharmacophore has been found, its structure is modelled according to its physical properties, e.g. stereochemistry, bonding, size and/or charge, using data from a range of sources (spectroscopic techniques, X-ray diffraction data and NMR). Computational analysis, similarity mapping (which models the charge and/or volume of a pharmacophore, rather than the bonding between atoms) and other techniques can be used in this modelling process.

In a variant of this approach, the three-dimensional structure of the ligand and its binding partner are modelled. This can be especially useful where the ligand and/or binding partner change conformation on binding, allowing the model to take account of this in the design of the mimetic.

A template molecule is then selected onto which chemical groups that mimic the pharmacophore can be grafted. The template molecule and the chemical groups grafted on to it can conventionaly be selected so that the mimetic is easy to synthesize, is likely to be pharmacologically acceptable, and does not degrade *in vivo*, while retaining the biological activity of the lead compound. The mimetic or mimetcs found by this approach can then be screened to see whether they have the target property, or to what extent

they exhibit it. Further optimization or modification can be carried out to arrive at one or more final mimetics for *in vivo* or clinical testing.

In this context, methods described in the literature allow efficient screening of libraries for desired binding activities (see Pluckthun & Ge, Angew. Chem. Int. Ed. Engl. 30, 296-298 (1991); US patent nos. 5 733 731; 5 872 015; and 5 612 895 Further, algorithms for three dimensional data base comparisons are available in the literature, see e.g., Cooper, et al., J. Comput.-Aided Mol. Design, 3: 253-259 (1989) and references cited therein; Brent, et al., J. Comput.-Aided Mol. Design, 2: 311-310 (1988) and references cited therein. Commercial software for such searches is also available from vendors such as Day Light Information Systems, Inc., Irvine, Calif. 92714, and Molecular Design Limited, 2132 Faralton Drive, San Leandro, Calif. 94577.

Finally, the present invention also relates to methods of medical treatment wherein the substances, fragments and molecules according to the invention are used. In specific embodiments, the methods may be preventive. Further, the substances and nucleic acids according to the invention may also be used in gene therapy. For a review of gene therapy procedures, see Anderson, *Science* (1992) 256:808-813; Nabel and Felgner (1993) *TIBTECH* 11:211-217; Mitani and Caskey (1993) *TIBTECH* 11: 162-166; Mulligan (1993) *Science* 926-932; Dillon (1993) *TIBTECH* 11: 167-175; Miller (1992) *Nature* 357:455-460; Van Brunt (1988) *Biotechnology* 6(10): 1149-1154; Vigne (1995) *Restorative Neurology and Neuroscience* 8: 35-36; Kremer and Perricaudet (1995) *British Medical Bulletin* 51(1) 31-44; Haddada *et al.* (1995) in *Current Topics in Microbiology and Immunology* Doerfler and Böhm (eds) Springer-Verlag, Heidelberg Germany; and Yu *et al.*, *Gene Therapy* (1994) 1:13-26.

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Detailed description of the drawings

Figure 1 illustrates how peptide 14 inhibit the sequence specific DNA bin-
ding of the p53 protein. More specifically, figure 1A shows how Baculovi-
rus-produced p53 protein was incubated with 100 ng of synthetic p53-
5 derived peptides and tested for binding to the 32P-labelled BC oligonucleo-
tide containing a p53 consensus binding site in a band shift assay. Lane 1,
DNA binding of the p53 protein alone; lane 2, activation of the p53 specific
DNA binding by peptide 46, lanes 3, 4, 5, 6, and 8 - peptides 10, 11, 12,
13, and 15, respectively, do not affect DNA binding of p53. Lane 7, inhibi-
10 tion of the specific DNA binding by peptide 14. Figure 1B shows the DNA
binding of the GST-p53 fusion protein in the presence of activating antybo-
dy PAb421 (100 ng) and peptide 14 (100 ng) was assayed as in A, and the
data was quantitated using PhosphoImager. Lane 1 - latent GST-p53 pro-
tein do not bind DNA, lane 2 - PAb421antibody activated p53 DNA bin-
15 ding, lane 3 - peptide 14 inhibited the PAb421 activated DNA binding of
p53, whereas control peptide 10 did not affect the binding (lane 4). Figure
1C shows how the DNA binding of the GST-p53 fusion protein induced by
peptide 46 was assayed as in Figure 1A. Graphic representation of the data
obtained in band shift assays quantitated by PhosphoImager is presented.
20 Peptide 46 activated the DNA binding of GST p53 in a dose dependent
manner: lanes 2 - 5, 10 ng of peptide 46, lanes 6 - 10, 50 ng and lanes 10 -
13, 100 ng of peptide 46. An excess of peptide 14 over peptide 46 is requi-
red in order to inhibit the DNA binding of p53: lanes 3,7, and 11 - 10 ng of
peptide 14; lanes 4,8, and 12 - 50 ng of peptide 14, lanes 5, 9, and 13 - 100
25 ng of peptide 14. Lane 1 - GST-p53 does not bind DNA in the absence of
peptides.

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Figure 2 illustrates the DNA binding of the constitutively activated GST-p53-Δ30 fusion protein is inhibited by peptide 14. The specific DNA binding of the GST-p53-Δ30 protein was tested in a band shift assay as described in Fig.1A. Incubation of the GST-p53-Δ30 protein with 100 ng of peptide 14 prevented its sequence specific DNA binding (lane 3). Overlapping peptides 13 and 15 did not affect the DNA binding of GST-p53-Δ30 (lanes 2 and 4).

Figure 3 illustrates the interaction of the peptide 14 with the core and C-terminal domains of p53. More specifically, figure 3A shows the 32P-labelled peptide 14 retained on glutathione-Sepharose beads with immobilized GST-p53 proteins was analyzed by SDS polyacrylamide gel electrophoresis and autoradiography. The results of quantitation performed using PhosphoImager are presented. Peptide 14 bound to the full length GST-p53 protein (1-393), column 2, and to the GST-p53 (99-307) and GST-p53 (320-393) fusion proteins, columns 4 and 5, respectively. No significant binding to the GST protein alone or to the GST-p53 (1-100) protein was observed (columns 1 and 2, respectively). Figure 3B shows how native gel electrophoresis reveals complex formation between the 32P-labelled p53 (99-307) protein and peptide 14. Incubation of the p53 (99-307) protein with increasing amounts of peptide 14 (lanes 3 and 4, 100 and 300 ng, respectively) , but not with overlapping peptides 13 and 15 (lanes 2 and 5, 300 ng of each), resulted in a shift of the p53 (99-307) protein band on the gel. C and D, the binding of the peptide 14 to the labelled C-terminal (residues 320-393) and N-terminal (residues 1-100) p53 polypeptides, respectively, was assayed as in B. Lane 1 in C and D, migration of the polypeptide in the absence of synthetic peptides, lanes 2, 3, 4, and 5 - migration of polypeptides upon incubation with synthetic peptides 10, 11, 12, 13, 14,

and 15, respectively. Peptide 14 altered the migration of the C-terminal po-
lypeptide (C, lane 3).

Figure 4 illustrates that peptide 14 prevents the complexing of peptide 46

5   with the p53 core domain. More specifically, figure 4A shows how the 32P-
labelled peptide 46 was incubated with the GST-p53 protein and the pepti-
de/protein complexes were separated by native polyacrylamide gel elect-
rophoresis. Lane 1, labelled peptide 46 forms a complex with the GST-p53
fusion protein; lanes 2 and 3, incubation of GST-p53 protein with 100 and

10   200 ng of unlabelled peptide 14 inhibited the complex formation with la-
belled peptide 46 in a dose-dependent manner; lane 4, control peptide 10
(200 ng) did not affect peptide 46/p53 complexing. Figure 4B shows how
peptide 14 prevented complex formation of the GST-p53 (99-307) protein,
representing the p53 core domain with peptide 46. Biotinylated peptide 46

15   was immobilized on Streptavidin-coated Dynabeads and incubated with the
GST-p53 (99-307) protein in the presence of several p53-derived peptides.
The protein bound to peptide 46-loaded beads was analysed by Western
blotting using the p53 specific antibody PAb240. Lane 1, 50 ng of the
GST- p53(99-307) protein; lane 2 - GST-p53(99-307) protein bound to

20   peptide 46 in the absence of competing peptide; lane 5, in the presence of
peptide 14 (50 mg) the GST-p53(99-307) protein did not bind to peptide 46;
lanes 3,4, and 6 - incubation with 50 µg of peptides 12,13, and 15, respec-
tively, did not affect complex formation.

25   Figure 5 illustrates the inhibition of p53 transactivation function of p53 by
peptide 14. More specifiically, figure 5A shows how the PG-CAT reporter
construct was cotransfected into HeLa cells together with wild type p53 ex-
pression vector and 20 or 50 µg/ml of the synthetic 22-mer peptides deri-

20

ved from the p53 core domain. Representative results from three independent experiments are shown. Column 1, transfection with empty vector. Wild type p53 caused a 10-fold increase in CAT activity (column 2). Introduction of peptide 14 resulted in complete inhibition of the transactivation

5 function of the wild type p53 protein (lane 4, 50 µg/ml), whereas control peptide 21 did not inhibit p53 function (column 5). Figure 5B shows how peptide 14 was cotransfected with PG-CAT reporter plasmid, wt p53 expression plasmid and C-terminal domain-derived peptide 46. Peptide 46 caused a strong induction of wild type p53-mediated transactivation of the

10 CAT reporter gene in a dose dependent manner (columns 2 and 3, 5 and 20 µg/ml, respectively). Introduction of 50 µg/ml of peptide 14 abolished the stimulatory activity of peptide 46 and completely inhibited p53 function (columns 4 and 5, 5 and 20 µg/ml, respectively). Column 1, transfection with empty vector.

15

EXPERIMENTAL

Below, the present invention will be described in more detail by way of examples that are not intended to limit the scope of the invention in any way. All references given below and elsewhere in the present specification are

20 hereby included herein by reference.

Materials and Methods

Plasmids. The plasmids encoding the GST-human wild type p53 fusion protein and the deletion fusion proteins GST-p53(1-100), GST-p53 (99-

25 307), GST-p53(320-393) and GST-p53-Δ30 were described earlier (Balkalkin et al., 1995; Selivanova et al., 1996). The PG-CAT plasmid containing 13 repeats of a p53 consensus site (Kern et al., 1992) and wild

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type p53 expression plasmid was a gift from Dr. B. Vogelstein, John Hopkins Oncology Center.

Synthetic peptides. The 22-mer synthetic peptides 10-15 span residues 73-126 of the p53 protein. Each peptide is shifted by 8 amino acid residues relative to the previous peptide. Peptides were synthesized using the Merrifield solid phase method or purchased from Genosys (UK). Purification by HPLC was performed on a Super Pac pep-S column. The sequences of the peptides in one letter code is follows: GSYGFRLGFLHSGTAKSVTCTY (peptide14; SEQ ID NO 1); VPSQKTYQGSYGFRLGFLHSGT (peptide 13; SEQ ID NO 2); FLHSGTAKSVTCTYSPALNKMF (peptide 15; SEQ ID NO 3) and AIYKQSQHMTEVVRRCPHHERC (peptide 21; SEQ ID NO 4).

Transfections and CAT assays. Transactivation assays using p53-responsive promoter constructs linked to the chloramphenicol acetyl transferase (CAT) reporter gene (PG-CAT) were performed as described (Selivanova et al, 1997). p53 expression vectors pC53-SN3 and pC53-SCX3 (V143A) or control vector pL15TK (0,5 µg each) were cotransfected with a CAT reporter plasmid PG-CAT (2 µg) and ß-galactosidase expression vector pRSV ßgal (0,5 µg) with or without synthetic peptides (5-50 µg) using lipofectamine (Gibco BRL). CAT activity was assayed 48 hours post transfection. The efficiency of transfection was verified by measurment of ß-galactosidase activity in cell extracts.

DNA binding assay. The GST-p53 proteins and baculovirus-produced p53 protein were prepared as described (Selivanova et al., 1996; 1997). Band shift assays were performed in binding buffer containing 100 mM Hepes

pH 7.5, 50 mM KCl, 1 mg/ml BSA, 0.1% Triton X-100, 2 mM MgCl2 and
1 mM DTT essentially as described (Selivanova et al., 1996).

Protein-protein and peptide-protein interaction assays. Reactions for the
peptide-protein interactions analysed in the native gel mobility assay were
performed in a buffer used for the DNA binding assay and containing 2%
BSA. GST-p53 proteins and peptides were labelled with 32-P-γ-ATP
phosphorylation by heart muscle kinase (Sigma) under the conditions
described by manufacturer. Peptide-protein and protein-protein complexes
were resolved on a 4% native polyacrylamide gel electrophoresis and expo-
sed to X-ray film.

For the GST pull down assay, 32-P-labelled peptide 46 was incubated with
the GST-p53 proteins (100 ng) immobilized on glutathione-Sepharose at
20°C for 30 minutes in binding buffer. After subsequent washing with bin-
ding buffer, peptides retained on the beads were eluted by boiling in a
sample buffer and separated on a 15% SDS polyacrylamide gel. Quantita-
tion was performed using PhosphorImage analysis (Molecular Dynamics).

Results and discussion

Inhibition of specific DNA binding of p53 by peptide 14
According to the present invention, a panel of 22-mer overlapping synthetic
peptides spanning the whole p53 protein sequence have been screened for
their ability to affect the specific DNA binding activity of the full length
p53 protein. The binding of the baculovirus produced p53 protein to the 32-
P-labelled DNA oligonucleotide containing the p53 consensus binding site

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was studied in band shift assays in the presence of 50 pM of synthetic peptides. Two peptides were selected that affected the specific DNA binding of p53. One of them, peptide 46, spanning residues 361 to 382 of the p53 C-terminus, stimulated p53 specific DNA binding 50-100 fold, as described earlier (Selivanova et al., (1997): "Restoration of the growth suppressor function of mutant p53 by a synthetic peptide derived from the p53 C-terminal domain, *Nature Med.* 3, 632-639), Fig. 1A, lane 2). Peptide 14, on the contrary, inhibited the specific DNA binding of p53. Peptide 14 is derived from the core domain of the p53 protein and corresponds to residues 105-126. Addition of 50-100 pM of peptide 14, but not overlapping peptides 13 and 15, to the reaction mixture inhibited the specific binding of the baculovirus-produced p53 protein (Fig. 1A, lanes 3-5). To gain further insight into the inhibition of p53 by peptide 14, it was investigated whether peptide 14 can interfere with the activation of p53 specific DNA binding by the monoclonal antibody PAb421 or peptide 46. Peptide 14 inhibited the DNA binding of the GST-p53 fusion protein stimulated by the activating antibody PAb421 (Fig 1B). The specific DNA binding of the GST-p53 protein was examined in the presence of increasing concentrations of peptide 46 followed by the addition of increasing amounts of peptide 14. As illustrated in Fig. 1C, peptide 14 inhibited peptide 46-stimulated DNA binding of p53 in a dose-dependent manner. An excess of peptide 14 over peptide 46 is required in order to inhibit specific DNA binding of p53.

The inhibition of the specific DNA binding of p53 by peptide 14 could occur either through an interaction with the p53 C-terminal regulatory domain or by interfering with core domain DNA binding directly. In order to distinguish between these two possibilities, it was tested whether peptide 14 can block DNA binding of the GST-p53-Δ30 protein that is constitutively acti-

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vated by deletion of last 30 C-terminal residues corresponding to the nega-
tive regulatory domain (Hupp, T.R., D.W.Meek, C.A. Midgley, and D.P.
Lane. (1992) Regulation of the specific DNA binding function of p53. Cell
71, 875-886). The GST-p53-Δ30 protein binds to DNA very efficiently, as

5   we have shown before (Selivanova et al., (1996): "The single stranded DNA
end binding site of p53 coincides with the C-terminal regulatory region",
Nucl. Acids Res., 24, 3560-3567), Fig. 2, lane 1). Incubation of the GST-
p53-Δ30 protein with peptide 14, but not the overlapping peptide 13, inhi-
bited its constitutively activated specific DNA binding (lanes 2 and 3).

10   Thus, peptide 14 was not only capable of inhibiting activation of p53 by
PAb421 antibody or C-terminal peptide, but blocked DNA binding of the
core domain in the absence of the negative regulatory domain.

Peptide 14 interacts with the core and C-terminal domains of the p53 pro-
15   tein

Next we addressed the question of the mechanism by which peptide 14 in-
hibits the specific DNA binding of p53. Does peptide 14 bind to the p53
protein? If so, which region in the p53 protein does it bind to? We investi-
gated the binding of the 32-P-labelled peptide 14 to the GST-fusion proteins

20   representing the full length p53 protein (residues 1-393), and the core
(residues 99-307), N-terminal (residues 1-100), and C-terminal (residues
320-393) p53 domains. The amount of labelled peptide 14 retained on Glu-
tathione-Sepharose beads with immobilized p53 proteins was quantitated
using PhosphoImager. Peptide 14 binds to the full length p53 protein, since

25   it was retained on beads with immobilized GST-p53 protein, but not with
GST protein alone (Fig. 3A, columns 1 and 2). Peptide 14 was also retai-
ned on beads containing the p53 proteins representing the core and C-
terminal domains (columns 4 and 5), but not the N-terminal domain

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(column 3). Thus, peptide 14 can bind both the C-terminal and the core domains of p53.

This conclusion was further supported by the observation that peptide 14 can shift the position of the p53 core and C-terminal domains in a native gel mobility assay. Incubation of a 32-P-labelled protein representing the core domain of p53 (residues 99-307) with peptide 14 but not the overlapping peptides 13 and 15 caused a shift in protein mobility, so that the protein does not enter the gel (Fig 3B, lanes 2-5). Disappearence of the protein band on a native gel may result from the change in conformation and/or oligomerization state of the core domain complexed with peptide 14. The migration of the protein corresponding to the p53 C-terminal domain (residues 320-393) in a native gel was also affected by the peptide 14 (Fig. 3C). In contrast, peptide 14 did not have any effect on the mobility of the protein corresponding to the residues 1-100 of the p53 N-terminal domain (Fig. 3D). Taken into consideration our results that peptide 14 is capable of inhibiting of DNA binding of the p53 protein with the deleted C-terminal regulatory domain, we suggest that peptide 14 blocks p53 DNA binding through the interaction with the core domain. Peptide 14/core domain complexing may result in a change of the core domain conformation and/or oligomerization state, preventing DNA binding. Alternatively, peptide 14 may block the core domain DNA binding due to steric hindrance.

It has recently been demonstrated by the present inventors that the peptide 46, corresponding to residues 361-382 of the p53 C-terminus, binds to the core domain of p53 (Selivanova et al., submitted). To test whether peptide 14 will prevent the interaction of peptide 46 with the p53 protein, the GST-p53 protein and 32-P-labelled peptide 46 were incubated in the presence of

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non-labelled peptide 14 and the peptide/protein complexes were separated from unbound peptide on a native gel (Fig. 4). In the absence of GST-p53 protein, 32-P-labelled peptide 46 migrated at the bottom of the native gel (lane 1). Incubation with the GST-p53 protein shifted the position of pepti-

5   de 46 on the gel followed by the formation of two more slowly migrating bands (Fig. 4A, lane 2). The peptide 46/GST-p53 complexes disappeared upon incubation with peptide 14 in a dose dependent manner (lanes 4 and 5). In contrast, control peptide did not have any effect (lane 6). Thus, peptide 14 prevented the complex formation between the full length p53 protein

10 and peptide 46.

Furthermore, peptide 14 blocked the interaction between peptide 46 and the p53 core domain. Biotinylated peptide 46, immobilized on streptavidin-coated Dynabeads, efficiently precipitated the GST-p53 (100-300) protein

15 representing the p53 core domain (Fig. 4B, lane 2). The peptide 46/core domain binding is inhibited by peptide 14, but not by control peptides 13 and 15 (lane 4 and lanes 3 and 5, respectively). Peptide 14 may compete with the core domain for binding to peptide 46. Alternatively, the inhibition of peptide 46/core domain complexing by peptide 14 might occur due to

20 changes in the core domain conformation and/or oligomerization state which mask the site for the peptide 46 interaction.

Peptide 14 blocks transactivation function of p53 in living cells

Having established that peptide 14 can inhibit specific DNA binding of p53

25 *in vitro* through a direct interaction with the p53 protein, the question whether peptide 14 can inhibit p53 function in living cells was addressed. The transcriptional transactivation function of p53 in HeLa cells was examined in the presence or absence of peptide 14, using a p53-responsive

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CAT reporter plasmid, PG-CAT. (Kern, S. E., J.A.Pietenpol, S.Thagaligam, A. Seymour, K.W. Kinzler, and B. Vogelstein. (1992) Oncogenic forms of p53 inhibit p53-regulated gene expression. *Science* **256**, 827-832.) Lipofection of peptide 14, but not the control peptide 21 corresponding to resi-

5   dues 161-182 of p53, along with the wild type p53 expression vector and the p53-responsive PG-CAT plasmid in HeLa cells caused a significant decrease in p53-induced CAT activity (Fig.5 A, column 4). Addition of peptide 14 had no effect on the transfection efficiency as verified by ß-galactosidase (ß-gal) activity (data not shown).

10

It was subsequently examined whether peptide 14 could overcome the peptide 46-mediated activation of p53 in living cells, as it did *in vitro* (see above). Peptides 14 and 46 were introduced in HeLa cells along with the p53 expression vector and PG-CAT plasmid. As can be seen in Fig. 5 B, pepti-

15   de 14 completely inhibited p53-mediated transactivation of the CAT gene stimulated by peptide 46 (compare columns 3 and 4 with columns 5 and 6). Notably, an excess of peptide 14 over peptide 46 is needed for the inhibition of p53 function. These results correlate with the data obtained in gel shift experiments and demonstrate that peptide 14 can work both *in vitro*

20   and *in vivo* as an inhibitor of the specific DNA binding and transactivation functions of p53.
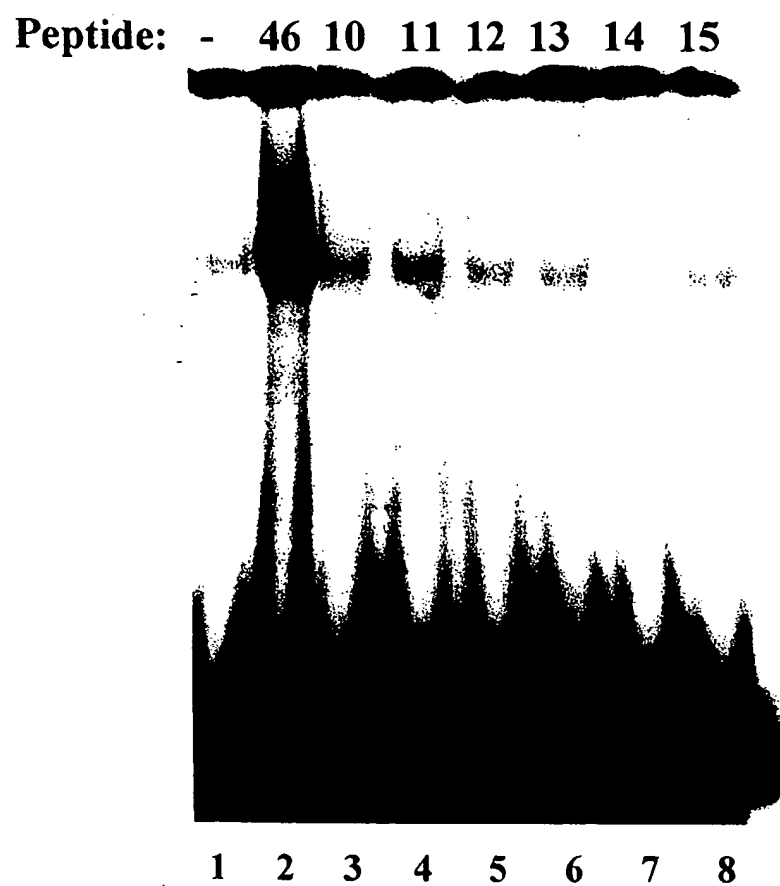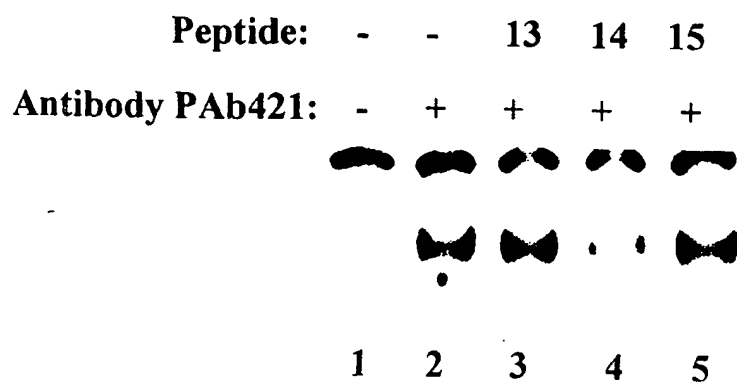
28

CLAIMS

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1. A substance which is capable of inhibiting the sequence specific DNA-binding activity of the p53 protein, characterised in that said inhibition is provided by binding to the core domains and/or C-terminal domains of p53 protein, which substance is comprised of a fragment of the amino acid sequence of the core domain of p53, or a derivative or a functional mimetic of said fragment, coupled to one or more functional moieties enhancing the p53 inhibiting effect of said substance.

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2. A substance according to claim 1, wherein said functional moiety is an additional p53 inhibitor.

3. A substance according to claim 1 or 2, wherein said fragment comprises a substantial part of the sequence disclosed in SEQ ID NO 1, preferably all of said sequence.

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4. A pharmaceutical composition comprising a substance according to any one of claims 1-3 and a pharmaceutically acceptable carrier.

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5. A composition according to claim 4, which further comprises one or more additional p53 inhibitors.

6. A substance according to any one of claims 1-3 for use as a medicament.

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7. Use of a substance according to any one of claims 1-3 in the manufacture of a medicament against a degenerative condition linked to enhanced deregulated apoptosis.

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8. Use according to claim 7, wherein the degenerative condition is ischemia or multiple sclerosis.

5   9. A nucleic acid encoding a peptidic substance according to any one of claims 1-3.

10. A nucleic acid which is capable of specificallly hybridising to a nucleic acid according to claim 9.

10

11. A vector comprising a nucleic acid according to claim 9 or 10.

12. A method of screening for compounds capable of inhibiting the sequence specific DNA-binding activity of the p53 protein, wherein a substance

15   according to any one of claims 1-3 is used.

13. A method of designing an organic compound capable of inhibiting the sequence specific DNA-binding activity of the p53 protein, wherein a substance according to any one of claims 1-3 is used as a lead compound.

20

14. A method according to claim 13, wherein the organic compound is modelled to resemble the three dimensional structure of amino acids no. 105-126 of p53 protein.
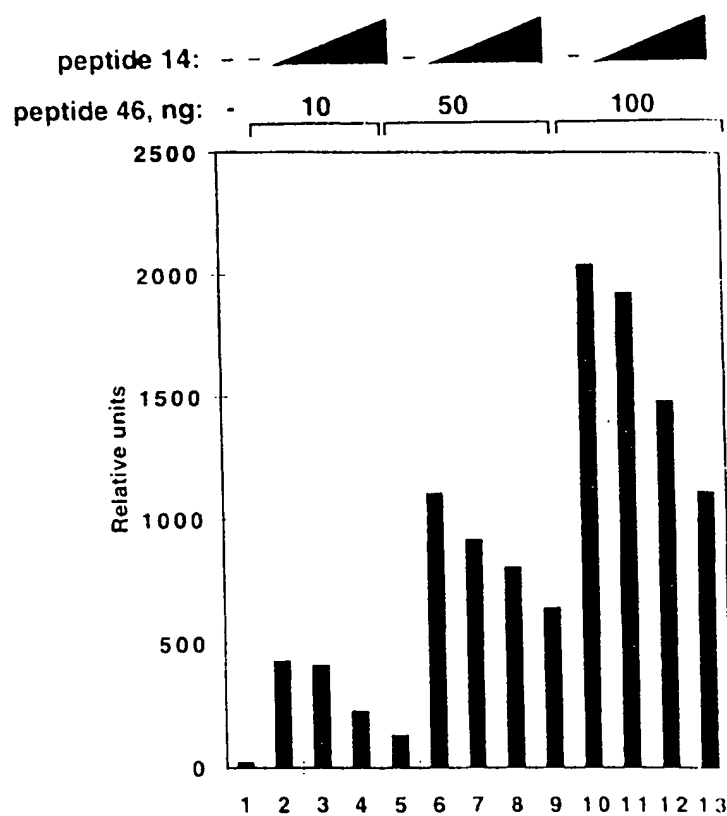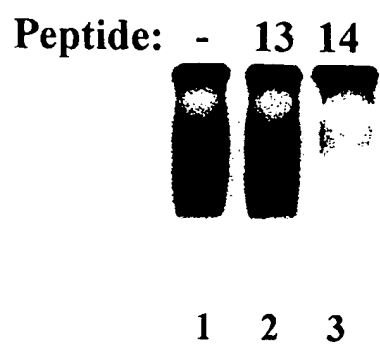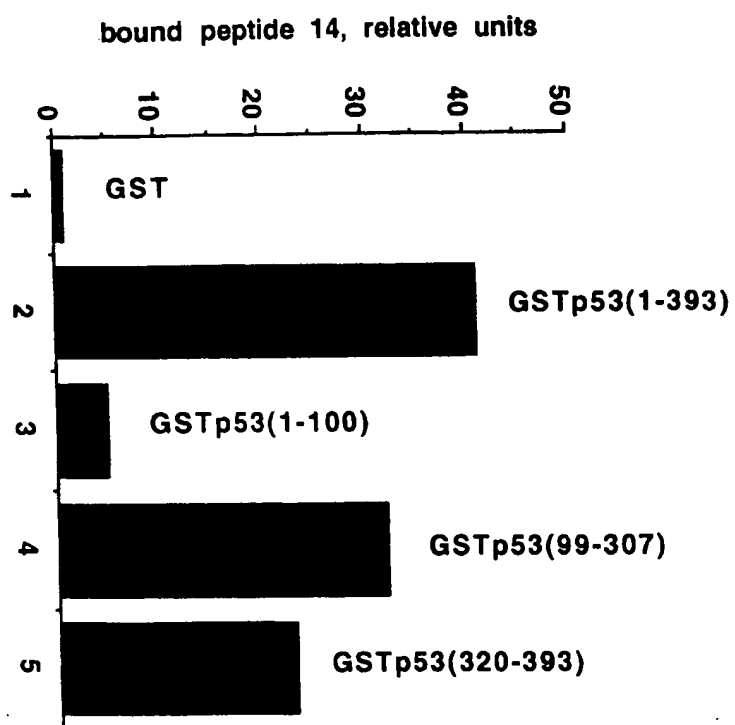
Fig 1A

Peptide: - 46 10 11 12 13 14 15
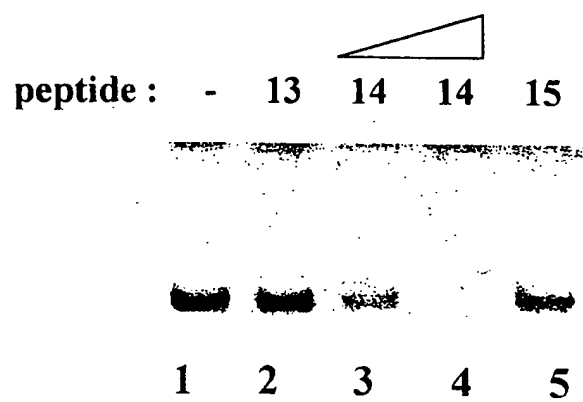
1 2 3 4 5 6 7 8

Fig 1B

Peptide: -   -   13   14   15

Antibody PAb421:   -   +   +   +   +

                   1   2   3   4   5

Fig 1C

Fig 2

Fig 3A

bound peptide 14, relative units

Fig 3B

Fig 3 C

Peptide: - 13 14

1 2 3
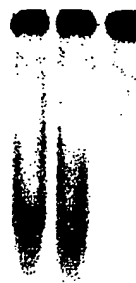
Fig 3 D
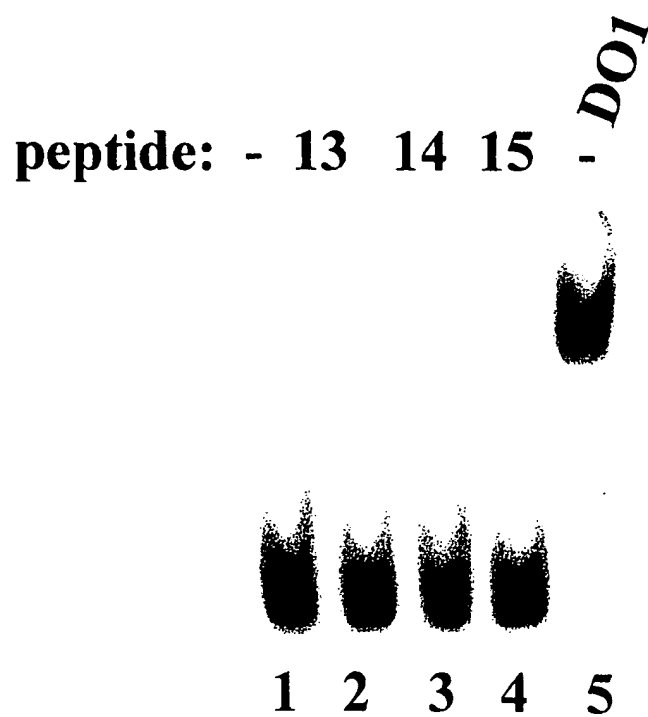
Fig 4A

A

peptide 15, ng	-	-	100	-	-	-	100	-	-
peptide 14, ng	-	100	-	-	100	200	-	-	200
p53, ng	-	-	-	10	10	10	10	20	20

free peptide 46 ➔

1 2 3 4 5 6 7 8 9

Fig 4B

Fig 5A

Fig 5B

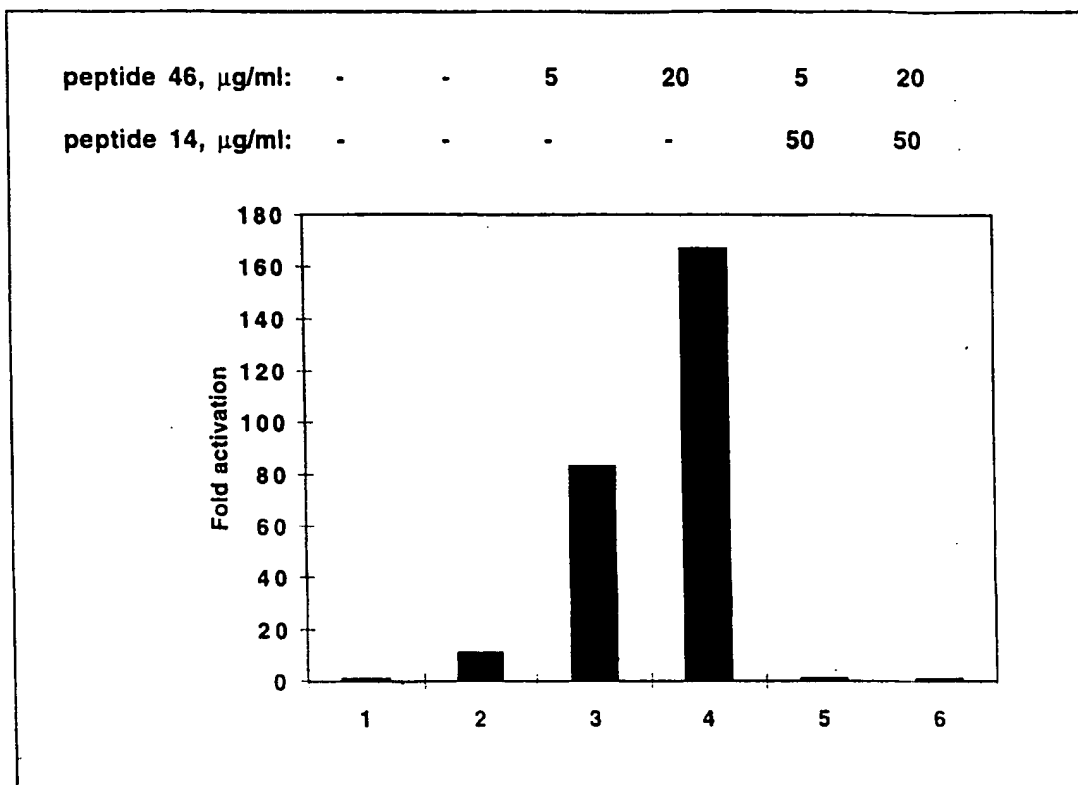| peptide 46, μg/ml: | - | - | 5 | 20 | 5 | 20 |
| peptide 14, μg/ml: | - | - | - | - | 50 | 50 |
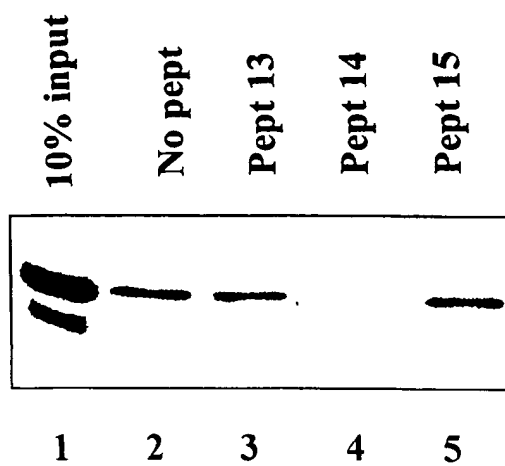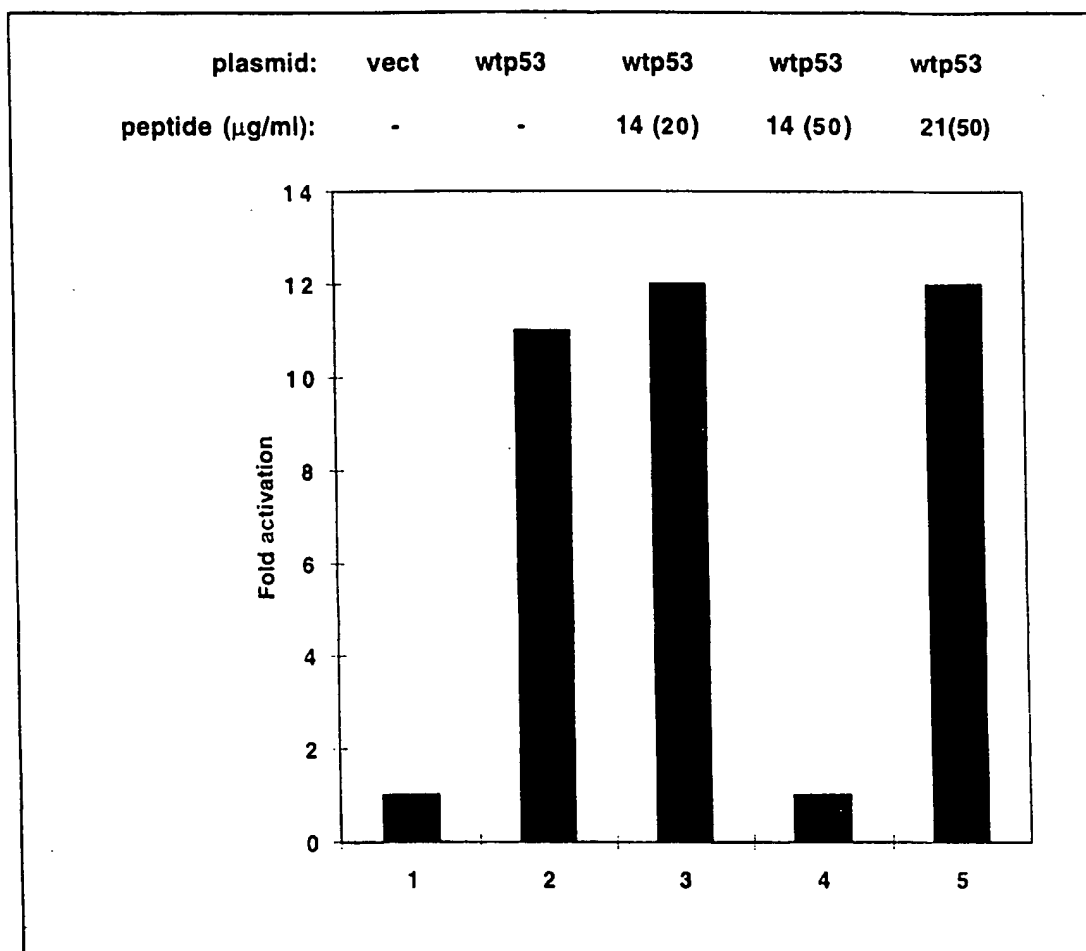
SEQUENCE LISTING

(1) GENERAL INFORMATION:

   (i) APPLICANT:
      (A) NAME: KAROLINSKA INNOVATIONS AB
      (B) STREET: KAROLINSKA INSTITUTET
      (C) CITY: STOCKHOLM
      (E) COUNTRY: SWEDEN
      (F) POSTAL CODE (ZIP): 171 77

   (ii) TITLE OF INVENTION: p53-DERIVED PEPTIDE

   (iii) NUMBER OF SEQUENCES: 4

   (iv) COMPUTER READABLE FORM:
      (A) MEDIUM TYPE: Floppy disk
      (B) COMPUTER: IBM PC compatible
      (C) OPERATING SYSTEM: PC-DOS/MS-DOS
      (D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

   (i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 22 amino acids
      (B) TYPE: amino acid
      (C) STRANDEDNESS: single
      (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: peptide

   (v) FRAGMENT TYPE: internal

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

Gly Ser Tyr Gly Phe Arg Leu Gly Phe Leu His Ser Gly Thr Ala Lys
1   5   10   15

Ser Val Thr Cys Thr Tyr
            20

(2) INFORMATION FOR SEQ ID NO: 2:

   (i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 22 amino acids
      (B) TYPE: amino acid

            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

    Val Pro Ser Gln Lys Thr Tyr Gln Gly Ser Tyr Gly Phe Arg Leu Gly
    1   5   10   15

    Phe Leu His Ser Gly Thr
                20

(2) INFORMATION FOR SEQ ID NO: 3:

    (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 22 amino acids
            (B) TYPE: amino acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

    Phe Leu His Ser Gly Thr Ala Lys Ser Val Thr Cys Thr Tyr Ser Pro
    1   5   10   15

    Ala Leu Asn Lys Met Phe
                20

(2) INFORMATION FOR SEQ ID NO: 4:

    (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 22 amino acids
            (B) TYPE: amino acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Ala Ile Tyr Lys Gln Ser Gln His Met Thr Glu Val Val Arg Arg Cys
1   5   10   15

Pro His His Glu Arg Cys
          20

INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 99/00807

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C07K 14/47, A61K 38/17

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C07K, A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 9519367 A1 (LA JOLLA CANCER RESEARCH FOUNDATION), 20 July 1995 (20.07.95), page 17, line 30 - page 18, line 31, claim 10	1-2,4-5,8-13
A		3,6
	--	
X	WO 9324525 A1 (RIJKSUNIVERSITEIT LEIDEN), 9 December 1993 (09.12.93), claim 3, SEQ IDNO:9	1-4,10-11
	--	
X	WO 9807851 A2 (BERGMANN, JOHANNA, E.), 26 February 1998 (26.02.98), figure 1a, claim 1	10
	--	

[X] Further documents are listed in the continuation of Box C.   [X] See patent family annex.

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	
"E" erlier document but published on or after the international filing date	"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
31 August 1999	2 2 -09- 1999
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Patrick Andersson/Eö Telephone No. +46 8 782 25 00

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 99/00807

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 9806753 A2 (TRUSTEES OF PRINCETON UNIVERSITY), 19 February 1998 (19.02.98), page 2, line 18, claims 1, 14	1-14

INTERNATIONAL SEARCH REPORT

Information on patent family members

: 02/08/99

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
WO	9519367 A1	20/07/95	US	5484710 A	16/01/96
			US	5659024 A	19/08/97
			US	5908750 A	01/06/99
WO	9324525 A1	09/12/93	AU	681853 B	11/09/97
			AU	4359193 A	30/12/93
			CA	2136624 A	09/12/93
			EP	0643726 A	22/03/95
			JP	8501280 T	13/02/96
			US	5679641 A	21/10/97
WO	9807851 A2	26/02/98	AU	5939898 A	06/03/98
			CA	2183900 A	23/02/98
WO	9806753 A2	19/02/98	AU	4394697 A	06/03/98